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Prevalence of human herpesvirus U94/REP antibodies and DNA in Tunisian multiple sclerosis patients

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Abstract Human herpesvirus 6 (HHV-6) has been linked to the pathogenesis of multiple sclerosis (MS). Based on antibody detection and quantitative HHV-6 polymerase chain reaction assay, this study aimed to analyze the possible association between infection with HHV-6 and MS. A total of 131 serum samples were analyzed by ELISA for the presence of specific antibodies to HHV-6 latency-associated U94/REP protein: 68 serum samples from 60 MS patients (20 in relapse and 48 in remission phase) and 63 serum samples from 63 healthy controls. Real-time quantitative PCR for HHV-6 U94/*rep* DNA was also performed in total blood of MS patients and healthy controls. The serological analysis by ELISA showed that MS patients had increased prevalence and titers of anti-U94/REP immunoglobulins in comparison with control group (seroprevalence 51.47 % versus 28.57 % and mean titer of positive samples 1:248 versus 1:110; $p=0.0005$), with significant difference between relapse and remission phases. HHV-6 DNA was detected in 4 of 60 MS patients (6.66 %) and in 2 of 63 healthy controls (3.17 %), confirming previous data of prevalence obtained by qualitative nested PCR. However, viral load was higher in MS patients compared to controls, and differences were statistically significant ($p=0.02$). The results show that, in spite of the low presence of HHV-6 DNA in peripheral blood, MS patients have increased prevalence and titer of IgGs reacting with HHV-6 latency-associated U94/REP protein.

Keywords MS · HHV-6 · U94/REP · Seroprevalence

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Introduction

Human herpesvirus 6 (HHV-6) is a β-herpesvirus first isolated in 1986 (Salahuddin et al. 1986). HHV-6 is characterized by an elective tropism for CD4+ T lymphocytes and macrophages, but the virus can infect several different cells, due to the virus cell entry receptor CD46, which is a ubiquitous protein expressed on the surfaces of many different types of human cells (Santoro et al. 1999). Consequently, the virus can infect a broad range of cells of different origin (Caserta et al. 2001), including cells of the central nervous system (CNS) (Chan et al. 2001), generally supporting low levels of replication.

Two variants of HHV-6 have been identified (HHV-6A and HHV-6B). They share extensive DNA homology but differ for epidemiology and disease association. HHV-6B is highly prevalent in the human population and is the etiologic agent of the childhood febrile disease exanthema subitum (roseola) (Yamanishi et al. 1988), while the HHV-6A variant has a lower prevalence and primary infection has not yet been associated to human disease (Braun et al. 1997). HHV-6 primary infection takes place in early childhood; thereafter, the virus persists in the organism, establishing a latent infection in peripheral blood mononuclear cells (PBMCs) characterized by the persistence of viral DNA in the absence of viral gene expression. The only viral transcript detected

in healthy donors during latency is U94, which has been proposed as a molecular marker for HHV-6 latency (Rotola et al. 1998).

The spectrum of diseases associated with or caused by HHV-6 has been enlarging; especially neurological complications are increasingly reported. Accumulating data have demonstrated a strong association of virus infection of the CNS with multiple sclerosis (MS). In fact, specific antigens of HHV-6 have been identified in plaques, and viral DNA has been detected both in neurons and in oligodendrocytes (Alvarez-Lafuente et al. 2006; Virtanen et al. 2005; Friedman et al. 1999; Challoner et al. 1995). Additionally, Akhyani et al. (2000) reported increased prevalence of HHV-6A in MS, and active HHV-6A infection has been detected in blood of patients with relapsing and remitting MS (Alvarez-Lafuente et al. 2004, 2006), as well as in their CSF cells (Rotola et al. 2004). Moreover, MS patients have increased titers of serum antibodies directed against HHV-6 (Ablashi et al. 2000; Sola et al. 1993), and 50 to 70 % of them are positive for HHV-6-specific immunoglobulin M (IgM) antibodies (Ablashi et al. 1998, 2000; Soldan et al. 1997). Nevertheless, the evidence is still controversial.

Caselli and co-workers set up an ELISA assay for the detection of antibodies specifically directed against U94/REP protein. They reported that the immune system of healthy donors is exposed to this antigen during natural infection and that MS patients show increased prevalence and titers of antibodies against HHV-6 U94/REP, suggesting that these patients might experience variations in U94 production, or frequent switches between latency and active replication, leading to an increased sensitization to this viral antigen (Caselli et al. 2002).

Recently, we have reported a low frequency of HHV-6 DNA presence in Tunisian patients affected with MS, as detected by nested PCR (Ben Fredj et al. 2012). In the present paper, we increased the number of patients and reanalyzed the same samples for the presence of HHV-6 DNA by real-time quantitative PCR, a very sensitive procedure allowing direct quantitation of virus. In addition, due to the previous results reported on the specific increase of anti-U94/REP antibodies in the Italian MS population, we investigated this aspect in the Tunisian population, analyzing both MS patients and healthy blood donors.

Materials and methods

Patients and samples

Sixty-eight blood samples from subjects with a clinical diagnosis of MS were obtained from 60 patients (22 males and 38 females), 12 of which were having an MS relapse when blood was drawn (48 h after onset of symptoms), 40 were in remission, and 8 MS patients were in both phases. This group was selected according to the criteria described by McDonald et al. (2001) and represented a cross-sectional population of patients; 28 patients were affected by relapsing–remitting MS and had received beta interferon therapy, whereas 32 were enrolled at an earlier stage of the disease and had not received any treatment. The mean age of MS patients was 38 years old (age range, 14 to 68). The mean age at disease onset was 32.81 years (range 16–58), the mean disease duration was 5.46 years (standard deviations (SD) 8.485, range 1–15), and mean Extended Disability Status Scale (EDSS) score was 2.921 (SD 1.414, range 1–8). Diagnostic criteria incorporate magnetic resonance imaging, cerebrospinal fluid, and evoked potentials testing. Disability was assessed with EDSS. Blood samples were collected at the Department of Neurology, Fattouma Bourguiba Hospital, Monastir, Tunisia.

For comparison, we had age- and sex-matched controls who had donated blood at the Regional Center for Blood Transfusion, Monastir, Tunisia; there were 35 men and 33 women (mean age, 34.26 years; age range, 19–49). Control blood samples were seronegative for HIV-1, HIV-2, hepatitis B virus, and hepatitis C virus obtained from 63 healthy blood donors.

The cohorts were established with the approval of the local Ethics Committee, and all the participants gave informed consent before the examination.

All of the samples were aliquoted and frozen at −80 °C until needed in order to avoid repeated freezing–thawing cycles.

ELISA assay

A total of 131 serum samples were analyzed by ELISA for the presence of antibodies specifically directed against HHV-6 U94/REP protein, as previously described (Caselli et al. 2002). They included 68 serum samples from 60 MS patients (20 in relapse and 48 in remission phase) and 63 serum samples from 63 healthy control. The source of antigen was HHV-6 U94/REP protein produced in recombinant bacteria (Caselli et al. 2002).

In brief, immunoplates (Nunc) were coated overnight at 4 °C with 5 μg/ml of purified recombinant U94/REP, or mock lysate, suspended in 0.05 M sodium bicarbonate buffer (pH 9.6). Excess of antigen was eliminated by three washings with phosphate-buffered saline (PBS) containing 0.05 % Tween 20 (PBS-T). A saturation step was performed by incubating plates for 90 min at 37 °C with 200 μl/well of a PBS solution containing 10 mM $CaCl_2$ and 5 mM $MgCl_2$ (PBS-C) and 3 % of bovine serum albumin (BSA, Sigma). After three washings with PBS-T, 100 μl of serum diluted in saturation buffer (PBS-C plus 3 % BSA) was added and tested in duplicate. Human sera previously tested and

resulted negative or positive for the presence of anti-U94/REP IgG (Caselli et al. 2002) were used respectively as positive and negative controls. Incubation was performed for 90 min at 37 °C. Plates were further washed three times with PBS-T then 100 μl of horseradish peroxidase-labeled goat anti-human IgG (Roche Molecular Biochemicals), diluted 1:10,000 in PBS containing 0.1 % Tween 20 and 1 % BSA, was added per well to reveal specific anti-U94/REP antibodies. Incubation was performed for 90 min at room temperature. Following three additional washings, 100 μl/well of ABTS substrate (Roche Molecular Biochemicals) was added for 45 min at room temperature. The absorbance was measured at 405 nm. Absorbance values higher than the mean negative control plus 3 SD were considered positive.

DNA extraction and real-time PCR

After collection, DNA was extracted from 200 μl of total blood using spin column technique of QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions, and eluted in 100 μl of water. Two negative controls, consisting of reagents only, were processed with each set of ten samples.

Real-time quantitative PCR for HHV-6 DNA was performed as described (Caruso et al. 2009), with the following set of primers/probe: HHV6 U94(+) (5′-GAGCGCCCGATAT TAAATGGAT-3′); HHV6 U94(-) (5′-GCT TGA GCG TAC CAC TTT GCA-3′); HHV6 U94 PROBE (5′-FAM-CTG GAA TAA TAA AAC TGC CGT CCC CAC C-TAMRA-3′). The standard curve was generated by amplification of a plasmid containing the targeted HHV-6 sequences. The method had a 6-log dynamic range and a sensitivity of 20 copies/sample. HHV-6 variants were characterized by restriction enzyme cleavage, as previously described by Di Luca et al. (1996).

Statistical analysis

The results were analyzed through standard statistical methods applied in case–control studies. The Student's *t* test was used to compare continuous variables with a p value of <0.05, considered as significant. An ANOVA test was performed to determine statistical significant between the mean titers and viral load values.

Results

To determine the presence and titer of circulating anti-U94/REP IgG in MS patients and healthy controls, 131 sera were analyzed by ELISA. All sera were serially diluted (1:50 to 1:2,700) and tested in duplicate against a purified U94/REP protein obtained in transformed bacterial cells (Caselli et al. 2002) or against a mock protein lysate, derived from bacterial cells transformed with the empty vector used for U94/*rep* gene cloning. Negative controls were represented by human sera which were previously shown to be negative for the presence of anti-U94/REP antibodies (Caselli et al. 2002). Cutoff values were calculated as the mean of negative control values plus 3 standard deviation values. The results are shown in Table 1. In the control group of healthy donors, 14 out of 63 samples were found positive for the presence of specific anti-U94/REP antibodies, with a seroprevalence of 28.57 %. No serum reacted against the mock lysate, confirming the specificity of the ELISA test used. The mean titer of positive samples was 1:110 (range, 1:50 to 200; median titer, 1:100±62.56).

By contrast, 35 of 68 sera derived from MS patients (51.47 %) showed a marked IgG response to U94/REP protein, with titers ranging from 1:50 to 1:2,700 (mean titer of positive samples, 1:1,248; median titer, 1:800±1,230.28). The difference in the fraction of positive samples between healthy controls and MS patients was statistically significant (p=0.0005).

Statistical analysis did not reveal any association between the presence of the titer of IgG anti-U94/REP and gender, age of onset, score, or beta interferon treatment. However, it is interesting to note that in eight MS patients, in which it was possible to analyze blood samples corresponding to both remitting and relapsing phases, the titer of anti-U94/REP IgG was clearly increased during the relapse (Table 2), and the differences observed were statistically significant (p=0.007).

Regarding the amplification results of HHV-6 DNA isolated from total blood using real-time PCR, HHV-6 DNA was detected in 4 of 60 MS patients (6.66 %) and in 2 of 63 healthy controls (3.17 %), thus confirming previous results obtained by qualitative nested PCR (3/51, 5.88 %) (Ben Fredj et al. 2012).

All of them were variant B, as determined by endonuclease cleavage of PCR products by HindIII and HinfII. We did not find any significant differences (p=0.36) between the

Table 1 Prevalence of HHV-6 U94/REP antibodies by ELISA analysis in the two groups of subjects: multiple sclerosis (MS) and healthy blood donors (BD)

Sample dilutions	MS (n=68)	HBD (n=63)	p value[a]
1:50	35 (51.47 %)	14 (22.22 %)	0.00054
1:100	25 (36.76 %)	9 (14.28 %)	0.0033
1:200	23 (33.82 %)	4 (6.34 %)	0.0001
1:400	21 (30.88 %)	0 %	0.0001
1:800	18 (26.47 %)	0 %	0.0001
1:1,200	15 (22.05 %)	0 %	0.0001
1:2,700	14 (20.58 %)	0 %	0.0001

[a] p values was calculated using χ^2 test

Table 2 Anti-U94/REP IgG titer comparison of eight MS patients who where both in relapse and remitting phase

Patients	Relapse phase titer	Remission phase titer	*p* value[a]
1	>1:2,700	1:50	
2	>1:2,700	1:800	
3	>1:2,700	1:400	
4	1:400	1:50	
5	>1:2,700	1:100	
6	1:400	Negative	
7	1:800	1:100	
8	1:200	Negative	
Mean titer	1:1,575	1:187	$p=0.007$

[a] *p* value was calculated using ANOVA test

presence of viral DNA in total blood samples of MS group and healthy controls. However, a larger sample size is needed for better evaluation.

With respect to viral load in total blood samples of MS patients, HHV-6 DNA copy number ranged from undetectable to 708 copies/ml. The lowest HHV-6 DNA molecules (85 copies/ml) were found in healthy controls and the highest HHV-6 DNA copy numbers (708 copies/ml) were found in the MS patients. Overall, HHV-6 viral load mean in MS patients was higher than in healthy controls: 368 versus 70 copies/ml and the differences observed were statistically significant ($p=0.02$).

Discussion

The first description in 1995 that HHV-6 might be associated to MS (Challoner et al. 1995) stirred considerable interest and prompted several studies to confirm the association and to elucidate a possible viral role. Several years have elapsed and the situation remains undetermined. Some studies confirmed viral findings in MS patients, other investigations failed to indicate a viral involvement.

The use of several techniques, with different sensitivities and specificities, might in part be responsible for the discordant results as well as the difference between patient's populations. Anyway, it is always difficult to establish etiologic associations when the viral agent infects the majority of the population, when it establishes latent infections, and when it has a low pathogenetic potential in the immunocompetent host.

As we recently found (Ben Fredj et al. 2012), HHV-6 in the Tunisian population has a very low prevalence, both in healthy controls and in multiple sclerosis patients. In addition, no association was observed within the MS patients in terms of gender, type of diagnosis, symptoms, disease score, and beta interferon treatment.

This has prompted us to study the serological profile of MS patients and healthy controls against U94/REP. Previous papers showed in fact that MS patients have increased prevalence and titer of antibodies directed against this specific virus protein (Caselli et al. 2002), while there is no significant variation in the general response against the whole virus. This is according with the fact that HHV-6 is a ubiquitous virus and its infection is widespread (Chua et al. 1996; Yoshikawa et al. 1989; Linde et al. 1988; Okuno et al. 1989).

Similar to what is observed in Italian population, the serological analysis of Tunisian MS patients by ELISA showed that they had increased prevalence and higher titers of anti-REP immunoglobulins in comparison with control groups (seroprevalence 51.47 versus 28.57 % and mean titer of positive samples 1:1,248 versus 1:110; $p=0.0005$).

To explain the high anti-U94/REP HHV-6 antibody titers in MS, it may be speculated that in this disease the impairment of the cellular immune response may lead to the reactivation of HHV-6 latent infection. This reactivation may in turn determine an increase of anti-HHV-6 antibody titers. This even in the absence of detectable viral sequences, since in our hands only 4 out of 60 MS patients were found positive for the detection of HHV-6 sequences in the total blood.

With the real-time PCR assay, only six positive cases were found, four of which were MS patients (6.66 %), and all of them were also positive for anti-U94/REP antibodies with elevated titers 1:2,700. Two positive cases were healthy controls (3.17 %), and these subjects were found negative for the presence of specific anti-U94/REP antibodies.

Our real-time PCR results are compatible with previously published data on the prevalence of HHV-6 DNA by nested PCR in these same samples from the Tunisian population, where we observed a low prevalence of HHV-6 in both MS patients and healthy controls, with no significant difference between MS patients (5.8 %) and healthy controls (1.9 %) (Ben Fredj et al. 2012). However, although prevalence percentages were very low, MS positive samples showed a higher virus load compared to controls (708 copies/ml versus 85 copies/ml). Due to the low number of positive subjects, the difference was not statistically significant, but it is suggestive of some increase of HHV-6 presence in MS patients, as recently suggested by Rizzo et al. (2012)

Several studies reported a higher prevalence of HHV-6 DNA both in MS patients and in healthy controls but no correlation between the virus and the disease was observed (Rotola et al. 2000; Kim et al. 2000; Âlvarez-Lafuente et al. 2002). The low prevalence of HHV-6 in our study might be a feature characteristic of the North African-specific geographical area (Ranger et al. 1991).

By contrast, the search of a specific immune response directed against the U94/REP antigen revealed unexpected significant results, confirming the value of this serological test to evidence an antibody reactivity which seems peculiar

to MS patients. In addition, in the present study, eight MS patients who where both in relapse and remitting phase had anti-U94/REP IgG, and interestingly, the titers in the relapse phase were higher than in remission phase with statistically significant differences (p=0.007). Further studies analyzing both phases (relapse and remission) of each MS patients are needed to better elucidate the involvement of HHV-6 in the pathogenesis of MS.

It is interesting to mention that since we reported an increased prevalence and titer of IgGs reacting with HHV-6 latency-associated U94/REP protein associated with very low prevalence of HHV-6 in MS patients, the analysis of the presence of U94/REP-specific IgMs may be of interest, mainly because previous study reported an increased prevalence and titers of HHV-6-specific IgMs in the MS patients (Ablashi et al. 1998, 2000; Friedman et al. 1999; Ongradi et al. 1999; Soldan et al. 1997). It would be also interesting to check the eventual presence of increased humoral or cellular responses directed against other virus proteins. Nevertheless, we recently reported that also in another HHV-6-related autoimmune disease (i.e., Hashimoto's thyroiditis), no variation is detected in the immune responses directed against other virus antigens, whereas both humoral and cellular anti-U94 responses are specifically increased (Caselli et al. 2012), suggesting that this virus antigen is specifically correlated to uncontrolled virus reactivations linked to the development of autoimmune diseases. Due to these results, a few sera from our MS patients were analyzed, obtaining similar results (Caselli et al., personal observations) and confirming our hypothesis.

The apparent discrepancy between serological and molecular data might be explained by the fact that viral DNA footprints can only be detected if the copy number is sufficiently high to be over the sensitivity threshold, whereas antibodies become increased each time that virus reactivates and a specific subset of memory cells is activated, and this increase remains visible even in the absence of detectable amounts of persisting virus.

The observed difference between the two populations (MS versus controls) could be related to the immune impairment of MS, which would make possible the expansion of a latent infection not allowed by an intact immune system. Otherwise, the immune impairment of the disease could be viewed as a consequence rather than as the origin of the high-viral titers.

Since HHV-6 is able to infect other cells beside T lymphocytes, a reason for these results may be that the virus remains latent in cells other than PBMCs like in the oligodendrocytes. To confirm this possibility, it might be relevant to study the presence of anti-U94/REP antibodies as well as HHV-6 DNA in the CSF of MS patients.

It is noteworthy that at present we cannot draw any conclusion in favor of or against a possible role of HHV-6 in the pathogenesis of some MS cases. Moreover, the possibility that HHV-6 proteins may cause the transactivation of other cellular or viral genes contributing in this way to the immune derangement of MS cannot be ruled out. We emphasize that further refining of antibody test systems, experience with larger numbers of subjects, and careful prospective and molecular studies are necessary before conclusions can be made regarding the relations between the MS and HHV-6 infection.

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Conflict of interest The authors declare that there are no conflict of interest policies.

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